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Sensitivity to Nd:YAG Induced Laserthermia Is a Cell-Type-Specific Feature Not Directly Related to Tumorigenic Potential or Proliferation Rate

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Background and Objective: **Laser-induced hyperthermia, laserthermia, is a promising new method for treating neoplasms. The response of different cell types to conventional hyperthermia varies [Bhuyan, Cancer Res 1979; 39:2277–2284; Raaphorst et al., Cancer Res 1979; 39:396–401]. We investigated the possible relationship between sensitivity to laser treatment and tumorigenic potential of three closely related cell types. Non-tumorigenic cells PYS-2 and differentiated F9S1 were compared to tumorigenic cells F9S1.**

Study Design/Materials and Methods: **The contact Nd:YAG laser was used in a continuous-wave mode with a power setting of 6W, exposure times were 2 and 4 min [Castrén-Persons et al., Lasers Surg Med 1991; 11:595–600; Castrén-Persons, unpublished data]. The frosted-end probe was placed in the middle of the well and a thermocouple was attached 5 mm from the tip of the probe. The total amount of energy was measured for each well. A 4 min, 44°C water bath treatment was used as comparison. Untreated wells served as controls. May-Grünwald-Giemsa staining and ^{3}H-thymidine labeling were used for the analysis.**

Results: **Laserthermia killed all three cell types significantly more effectively than the water bath. PYS-2 cells were the most sensitive to the laser treatment. At the same temperature, PYS-2 cells were only slightly affected by water bath induced heating; the differentiated F9 cells were the most sensitive to this treatment. During the laser treatments, the energy required for holding the temperature seemed to depend not only on the cell type but also on the amount of cells treated: the more cells in the well, the more energy was needed.**

Conclusion: **Our results suggest that laser sensitivity is a cell-type specific feature which is not directly related to the proliferation rate or benign or malignant behavior of the cells.**

Key words: embryonal carcinoma, F9 cells, PYS-2 cells, differentiation, laser, Nd:YAG, hyperthermia

INTRODUCTION

Hyperthermia has a tumor cell killing effect that has been known for centuries. Laser-induced hyperthermia has shown to be an even more effective killer of malignant cells [1]. Even though laserthermia is increasingly used and investi-

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gated in clinical work, its effects on cell and tissue levels have remained poorly characterized. The lack of basic knowledge of the effects of laserthermia may be reflected in the fact that the method is mainly used palliatively on patients out of the reach of curative treatment.

The response of neoplastic cells to heat (43°C water bath) has often been compared with the response of normal cells of the same histological type. In such studies, human melanoma cells were found to be more sensitive than human melanocytes, colon carcinoma cells more sensitive than fetal intestinal epithelial cells, rat sarcoma cells more sensitive than normal mesenchymal cells, and so on [2,3]. Several studies show that both human and animal cells grow more sensitive to heat upon transformation to neoplastic, tumor-producing cells [2,4,5]. Furthermore, the environment of cells affects their heat sensitivity: cells in different culture media show different sensitivity, and cells growing in monolayer are more sensitive than the same cells in suspension [2]. Growth rate also affects the heat sensitivity: some cells are more sensitive when growing exponentially [5] and some, in the plateau phase [2,6]. Mitotic and S-phase cells are more sensitive than G-phase cells [2].

Teratomas are tumors found in all vertebrates. Malignant teratomas, or teratocarcinomas, contain not only differentiated cells but also stem cells that proliferate in an uncontrolled way. These malignant stem cells are called embryonal carcinoma (EC) cells. Depending on environmental conditions, these cells can proliferate as undifferentiated malignant cells or differentiate into normal embryonic or adult type cells [7]. Consequently, EC cells have been widely used in experimental studies on differentiation and tumorigenesis.

In the present study, we investigated the relationship between laser sensitivity and tumorigenic potential of three closely related cell types, F9S1 and PYS-2 cell lines, both derived from the same transplantable teratocarcinoma tumor OTT6050, and experimentally differentiated F9S1 cells. The F9S1 EC cells are continuously growing malignant cells which form tumors when transplanted in syngeneic mice. The second cell type used in this study, PYS-2, is a non-tumorigenic parietal yolk sac endoderm cell line, which does not revert to carcinoma cells [8]. There are no studies on the proliferation rate of PYS-2 cells, but it seems to be fairly slow with a doubling time of approximately two days. The third cell type used in this study was the differentiated F9S1 cell. Retinoid treatment of F9 EC cells induces them to differentiate into endoderm-like (END) cells [9–13]. By combining dibutyryl cyclic AMP (dbc-AMP) to retinoic acid (RA) treatment, F9 cells can be made to differentiate towards parietal endoderm similar to the extraembryonic endoderm of the parietal yolk sac of the developing mouse embryo [14,15] (Fig. 1). During this process F9 cells lose their malignant characteristics [16,17] and become unable to form a tumor when transplanted in syngeneic mice [18]. Simultaneously, the cell cycle time of the cells doubles; this is due to the lengthening of G_1 and S phases [11,19]. In experimental F9 cell tumors in vivo, RA supplementation of the diet of tumor-bearing mice results in endodermal differentiation and growth arrest of the tumor cells [20]. Thus, RA treatment induces F9 cells to change from a nondifferentiated malignant phenotype into a differentiated benign phenotype both in vitro and in vivo.

MATERIALS AND METHODS

Cell Lines

The F9S1 EC cells and PYS-2 endoderm cells were cultured as previously described [9–12]. The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The medium was renewed daily.

To induce differentiation the F9S1 cells were plated on day 1 on gelatinized 10-cm dishes at a density of $2–5 \times 10^3$ cells/cm^2. On day 2, 5×10^{-8} M RA (Sigma Chemical Co., St. Louis, MO) and 10^{-3} M dbc-AMP (Sigma) were added [9,12]. The medium was changed every second day. In all, the cultures were treated for 5 days [11,21]. For the experiments, all the cell types were plated in gelatinized 48-well tissue culture plates (Costar^R, Cambridge, MA) at a density of $7–10 \times 10^4$ cells/cm^2 on the day preceding the treatments.

Laser

The contact Nd:YAG laser (CL 60, Surgical Laser Technology^R, Malvern, PA) was used in a continuous wave mode with a power setting of 6W. The frosted-end contact probe (MPR 2, SLT^R) was placed in the middle of the treated well. The temperature was monitored continuously using a digital thermometer with one thermocouple, diameter 0.5 mm (Exacon^R, Denmark), placed 5 mm from the tip of the contact probe. The thermocouple was physically attached to the contact probe

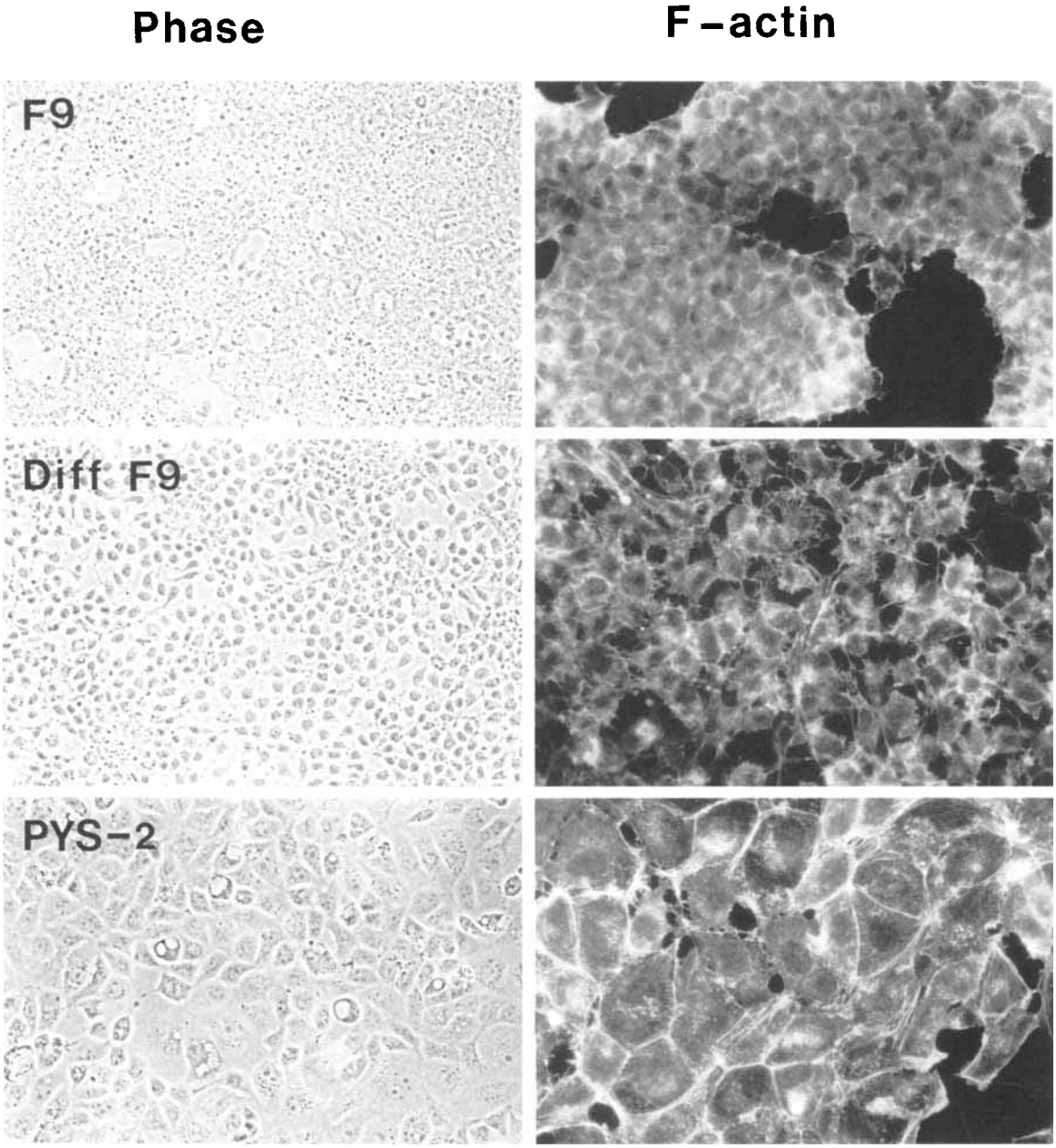

Fig. 1. The cell types used: undifferentiated and experimentally differentiated F9S1 cells and PYS-2 cells. The cells were plated on glass coverslips and fixed with paraformaldehyde. Left-hand panels are phase contrast micrographs, right-hand panels phalloidin stainings visualizing fibrillar actin [for technical details, see 9]. All cells are typical adherent cells, PYS-2 are largest in size and F9S1 smallest. The intercellular contacts between neighboring cells are most extensive in PYS-2 cells and least in differentiated F9S1 cells. Original magnifications in left hand panels are ×200, and in right hand panels × 400.

[1]. The diameter of each well was 10 mm. The total amount of energy delivered during the treatment was measured for each well.

Experimental Design

All of the experiments were carried out after replacing the media with 1 ml of prewarmed HEPES-buffered PB1/bovine serum albumin [22] immediately before the treatments. Immediately after the treatments, the cells were changed back to MEM supplemented with 10% FCS.

The laser treatment was compared with conventional hyperthermia. For the laser the exposure times were 2 and 4 min [1,23]. The temper-

TABLE 1. The Effect of Water Bath and Laserthermia on Cell Survival*

	Control	Laserthermia	
		2 min	4 min
Undifferentiated F9S1	69,658 ± 1,705	28,603 ± 11,006	1,678 ± 588
Differentiated F9S1	67,443 ± 993	33,297 ± 7,911	976 ± 544
PYS-2	97,709 ± 2,411	12,504 ± 12,033	315 ± 82

*The three cell types (undifferentiated F9S1, differentiated F9S1, and PYS-2) were exposed to two different laser treatments. The figures are counts representing radioactivity retained by cells surviving the treatment. Untreated wells served as controls. For each cell type, the three figures differ significantly from each other ($P < 0.001$).

ature was continuously measured as described above. Any well that even momentarily reached 44.5°C was excluded. A standard laboratory bath was used to apply conventional hyperthermia [1]. The cells were treated for 4 min at 44°C. In each experiment, untreated wells kept in room temperature served as controls. Each experiment included at least six wells for every variable and was repeated several times.

Cell Staining

The cells were stained with May-Grünwald-Giemsa 24 h after the treatment [24].

Metabolic Labeling

The relative quantities of cells were estimated 24 h after the treatments by labeling the cells with 1 μCi ml^{-1} of ^{3}H-thymidine (New England Nuclear, Boston, MA) in the culture medium for 12 h. The medium, containing the cells detached from the substratum, was removed by rinsing with PBS. The adherent cells were solubilized in 500 μl of 1% sodium dodecyl sulfate (SDS) in 0.5 N NaOH, and the radioactivity was counted in an LKB beta counter [1,13]. All figures were corrected by substracting the mean background measured in the wells without cells.

Statistics

The Kruskal-Wallis one-way analysis of variance was used for the statistical evaluation of the data. A P-value of < 0.05 was considered significant.

RESULTS

F9S1 Cells

As compared with untreated controls (100%), a 4-min treatment in a 44°C water bath did not affect the amount of viable F9S1 cells. A 2-min laser treatment at the same temperature killed 59% of the cells ($P < 0.001$) and a 4-min treatment, 98% of the cells ($P < 0.001$) (Table 1, Fig. 2). The difference between the 4-min and 2-min treatments was significant ($P < 0.05$). Laserthermia was more effective than the water bath in killing F9S1 cells after both a 2-min treatment ($P < 0.05$) and a 4-min treatment ($P < 0.01$).

Differentiated F9S1 Cells

The differentiated F9S1 cells were sensitive to all treatments. A 4-min water bath treatment killed 29% of the cells ($P < 0.07$) and a 2-min laser treatment, 51% ($P < 0.001$). The difference between these two treatments was not statistically significant. A 4-min laser treatment killed 99% of the cells. This treatment was very effective when compared with the results of the control wells ($P < 0.001$), the wells after a 2-min laser treatment ($P < 0.001$), or with those after the water bath treatment ($P < 0.01$) (Table 1, Fig. 2).

PYS-2 Cells

The PYS-2 cells were slightly affected by a 4-min incubation in a 44°C water bath: the amount of viable cells after the treatment was reduced by 7% from that in the control wells. The cell-killing effect of the laser, on the other hand, was strong: a 2-min treatment killed 87% of the cells ($P < 0.001$). A 4-min treatment killed practically all of the cells, 99.7% ($P < 0.001$) (Table 1, Fig. 2). There was no statistical difference between the two laser treatments. Laserthermia, both 2- and 4-min treatments, killed significantly more cells than did the water bath ($P < 0.01$).

Comparison of the Three Cell Types

The three cell types showed varying relative sensitivities to the two hyperthermia treatments (Fig. 2). The most sensitive to laserthermia was the PYS-2 cell: a 2-min treatment killed nearly all of the cells. Statistically, PYS-2 cells were more sensitive than the differentiated F9S1 cells to a 2-min laser treatment ($P < 0.05$), and they

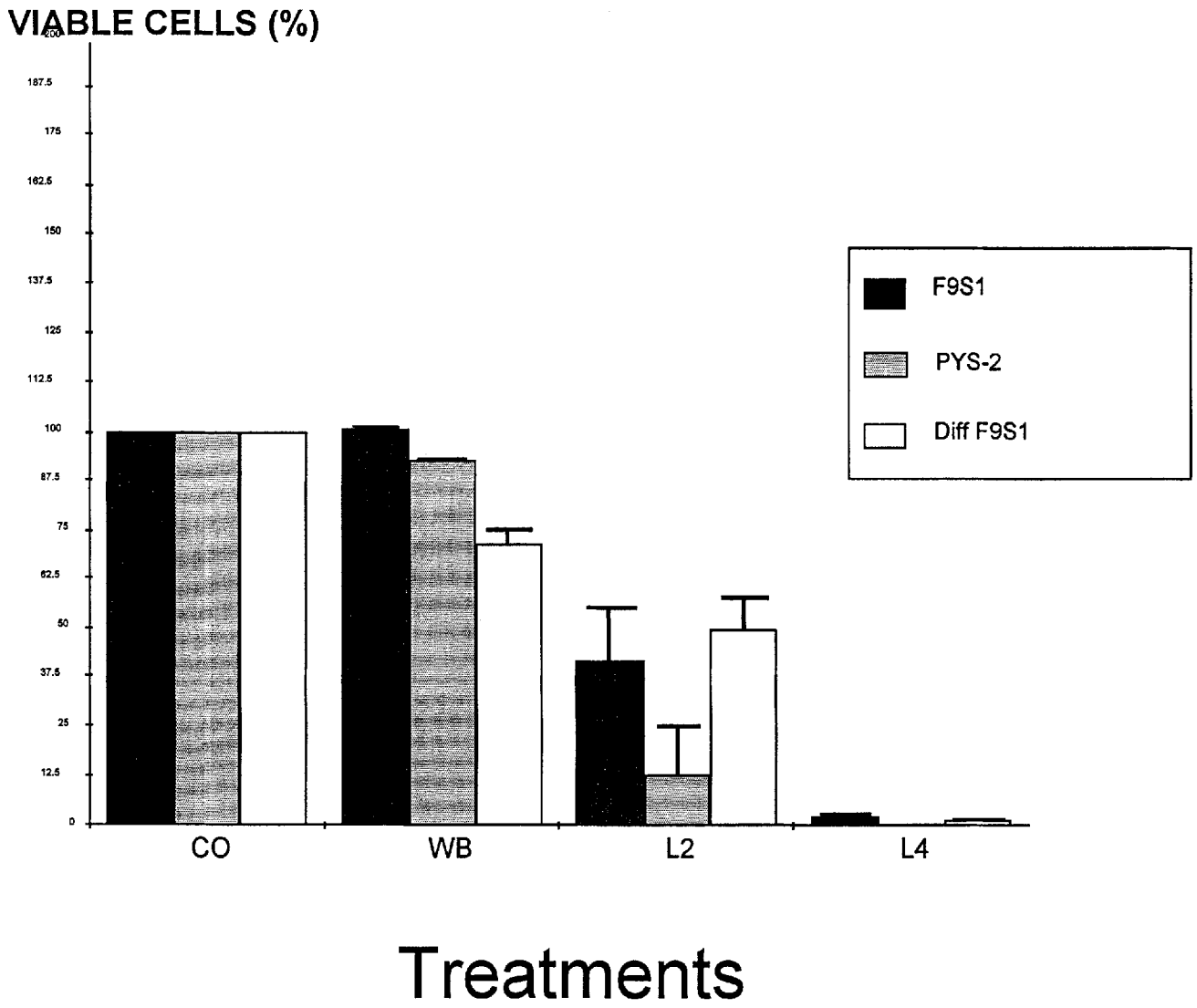

Fig. 2. The comparison of the 2- and 4-min laser treatments (L2 and L4) with the water bath (WB) when the tumorigenic F9S1 cells and the non-tumorigenic differentiated F9S1 and PYS-2 cells. F9S1 vs. PYS-2: L4 $P < 0.01$, WB $P < 0.01$; F9S1 vs. differentiated F9S1: L2 $P < 0.05$, WB $P < 0.01$; differentiated F9S1 vs. PYS-2: L2 $P < 0.05$; WB $P < 0.01$. Results are expressed as a percentage of the label in untreated controls (100%). Values are the means of six readings and the standard error of the mean (SEM).

were more sensitive than the undifferentiated F9S1 cells to a 4-min treatment ($P < 0.01$). Laserthermia, both 2- and 4-min treatments, had a very similar effect on both undifferentiated and differentiated F9S1 cells. To water-bath-induced hyperthermia, on the other hand, the differentiated F9S1 cells showed higher sensitivity than the undifferentiated cells ($P < 0.01$) or the PYS-2 cells ($P < 0.01$). PYS-2 cells were more sensitive than the undifferentiated F9S1 cells to water-bath-induced hyperthermia ($P < 0.01$). All of the cell types were extinguished after a 4-min treatment in a 59°C water bath (Fig. 3). Laserthermia killed all three cell types significantly more effectively than did the conventional water-bath-induced hyperthermia (Fig. 4).

The Energy Used in the Laser Treatments

The amount of laser energy needed for creating the desired temperature conditions varied between the different cell types. Lesser energy was needed for treating undifferentiated F9S1 cells than for treating PYS-2 or differentiated F9S1 cells. In the 4-min treatment the difference between F9S1 and both of the other cell types (F9S1 vs. PYS-2, $P < 0.05$ and F9S1 vs. differen-

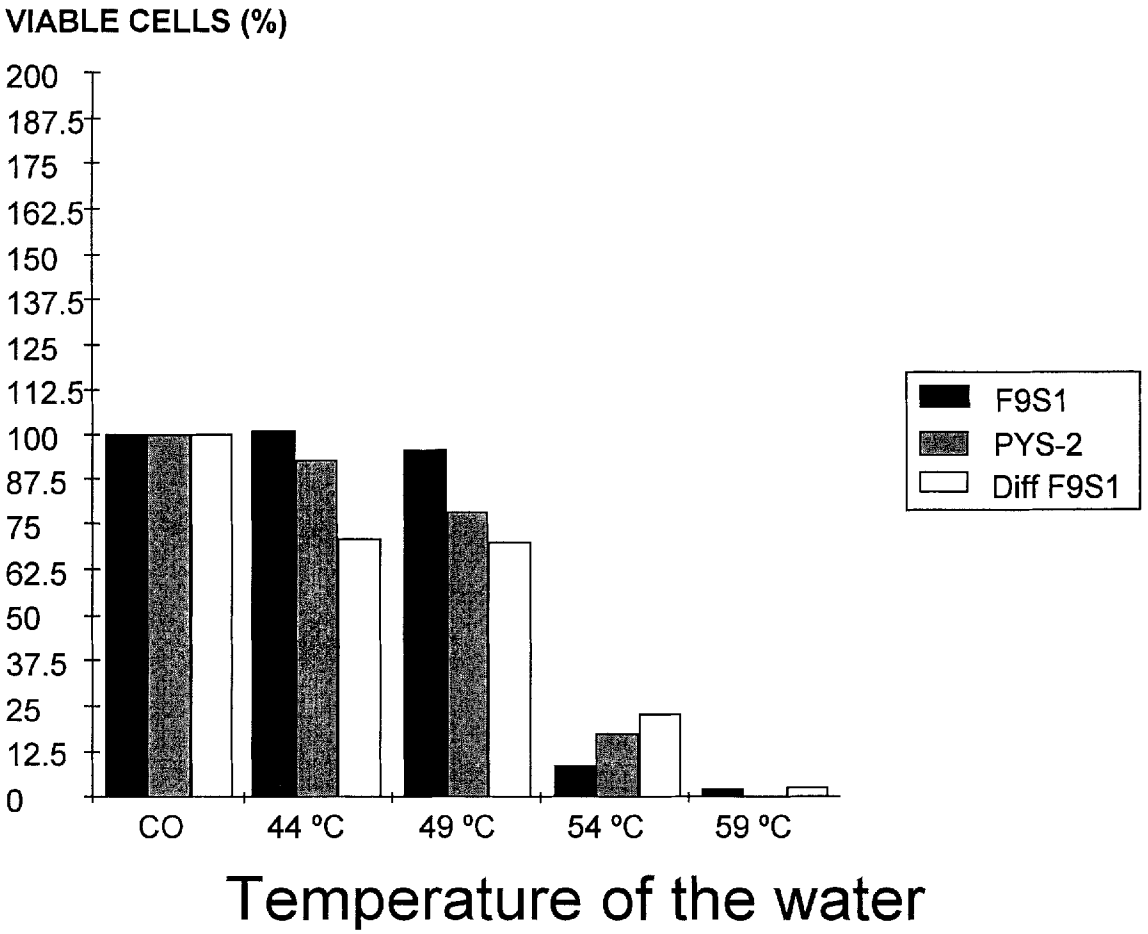

Fig. 3. The effects of water-bath-induced hyperthermia (37°C, 44°C, 49°C, 54°C, 59°C). The tumorigenic F9S1, the non-tumorigenic differentiated F9S1, and the PYS-2 cells were all extinguished at 59°C. The viability is expressed as a percentage of the counts in the control wells (37°C).

tiated F9S1, $P < 0.001$) was statistically significant (Table 2).

Our preliminary studies showed that the amount of laser energy needed for holding the 42–44°C temperature depended on the amount of cells in the well. When the amount of PYS-2 cells were doubled, the energy required was also doubled: a 2-min treatment of 40,000 cells needed 127 J and 90,000 cells 270 J, respectively. Similarly a 4-min treatment required 226 J and 443 J. After these preliminary studies, we kept the number of cells constant in each experiment.

DISCUSSION

The reason for launching this study was the observation that while laserthermia is increasingly used in clinical work, the knowledge of its effects on cellular and tissue level is minimal. A palliative method may be used this way but if we ever want to make this fairly non-invasive and very elegant method therapeutic, we have to know more about its effects. Specifically, we should be interested not only in the destruction of the center of the tumor but also in killing of the cells in the periphery of the tumor without damaging the neighboring nontumorous tissue. The aim of this study was to investigate whether nontumorigenic and tumorigenic cells show differential sensitivity to laserthermia.

It has been suggested that cells become more heat sensitive upon transformation to tumor-producing cells [4]. The present results on undifferentiated malignant EC cells and their differentiated counterparts, benign endoderm cells, disagree with this view and rather suggest that laser sensitivity is not directly related to the proliferation rate or benign or malignant behavior of

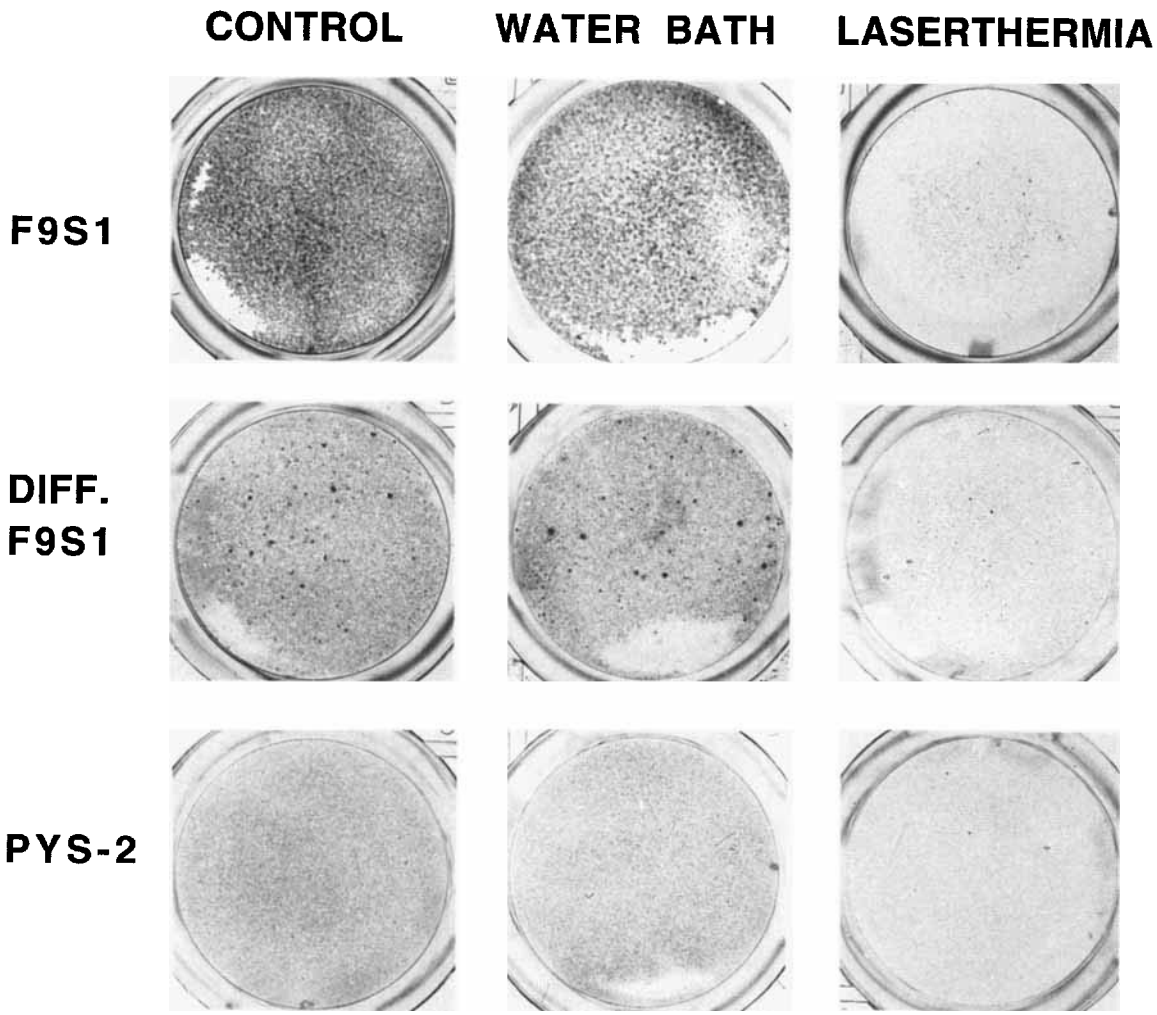

Fig. 4. The effect of laserthermia (42–44°C) and water bath (44°C) on tumorigenic F9S1 embryonal carcinoma cells, nontumorigenic differentiated F9S1 cells, and PYS-2 cells. Cells were seeded in 48-well plates, treated on the following day, cultured overnight and stained with the May-Grünwald-Giemsa method. In comparison with the untreated controls, a 4-min laserthermia treatment reduced the amount of all cells significantly. The water bath did not reduce the amount of F9S1 or differentiated F9S1 cells. The amount of PYS-2 cells was reduced only slightly.

the cells, but a cell-type-specific feature. The cells studied here differ in a number of features including size, the extent of intercellular contacts, distribution of F-actin [9a, the present study], the expression pattern of intermediate filaments [9–12], extracellular matrix molecules and cell surface structures [11], and adhesion properties [13].

Our preliminary results showed that when the amount of PYS-2 cells was doubled it was necessary to double the amount of energy to keep the temperature at 42–44°C for the desired time. This may mean that a cell can absorb a constant amount of laser energy before being damaged irreversibly, and thus the amount of laser energy needed for cell killing would be proportional to the number of cells treated. This finding may be clinically significant considering that different lesions consist of varying relative quantities of cell and matrix components. For the final data presented in the tables and figures of this study, the amount of cells as well as the culture conditions were standardized.

The laser settings used in this study were based on the results from our earlier experiments [1] in which we measured the temperatures in the well at different distances from the tip of the laser probe. In the present study, we focused on treating the well so that the cells most far away from the probe were heated to 43°C. This mimics the

TABLE 2. Energy Used When Inducing Hyperthermia With Nd:YAG Laser*

	Undifferentiated F9S1		Differentiated F9S1		PYS-2	
	2 min	4 min	2 min	4 min	2 min	4 min
Mean Energy (J)	201.7	306.3	241.7	390.7	270.3	443.0
SEM	8.9	11.5	15.2	17.7	10.8	22.7
Maximum	222.0	351.0	328.0	478.0	300.0	547.0
Minimum	165.0	278.0	169.0	304.0	225.0	385.0

*The energy required for holding the wells at 42–44°C during the treatment was measured. The PYS-2 cells needed significantly more energy during the 2-min treatment than the F9S1 cells ($P < 0.05$). In the 4-min treatment, the energy used for the F9S1 cells differed from that measured for the differentiated F9S1 cells for PYS-2 cells ($P < 0.05$). There was no difference between the differentiated F9S1 and PYS-2 cells.

clinical treatment where the margins of the tumor can not be treated with temperatures higher than 43°C.

Our results are in line with the finding that in the intestine, a hyperthermia-induced injury is similar in proliferating and post-mitotic non-proliferating cells [25]. Irradiation, on the other hand, killed only the proliferating intestine cells leaving the non-proliferating ones intact. In the present study, all three cell types were effectively killed by laserthermia at 42–44°C, PYS-2 being the most sensitive to the treatment.

In clinical trials, the histogenetic type of the tumor treated with laser-induced hyperthermia is usually mentioned, but whether or not it has had any correlation with the results of the treatment has not been discussed. Very little attention has overall been given to describing the histology of the tissue treated. This is rather surprising as we know that the conventional methods like radiation and chemotherapy affect different tissue types in a very different way. For optimizing the clinical use of laserthermia, it would thus be important to define the relative sensitivity of different cell types to this treatment.

We believe that in the future, laserthermia will be used as a therapeutic treatment for cancerous tumors in sites difficult or impossible to reach with the conventional surgical methods. Such locations typically include the brain and the vicinity of big vessels, where the treatment must be restricted to the neoplastic tissue as exclusively as possible. We need more knowledge of the effects of laserthermia on the zone between the tumor and healthy tissue since this determines whether the treatment was therapeutic, palliative, or harmful for the patient.

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